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Impact of Serum Amyloid A on Tissue Factor and Tissue Factor Pathway Inhibitor Expression and Activity in Endothelial Cells

Yulan Zhao, Shuli Zhou, Chew-Kiat Heng

Objective—Although serum amyloid A (SAA) is a useful biomarker of coronary artery disease (CAD), its direct role in procoagulation is obscure. This study investigates the impact of SAA on the expression and activity of tissue factor (TF) and tissue factor pathway inhibitor (TFPI) in endothelial cells.

Methods and Results—SAA was found to disturb the balance of TF and TFPI expression and activity in human endothelial cells. SAA (20 μ g/mL) markedly induced TF expression between 4 to 8 hours in both protein and mRNA levels, as well as TF activity. Conversely, incubation of SAA (20 μ g/mL) for 24 and 48 hours was found to significantly inhibit TFPI secretion, transcription, and activity. Pretreatment with formyl peptide receptor-like 1 (FPRL1) inhibitors (Pertussis toxin and WRWWWW) could block the SAA effects on TF and TFPI. Furthermore, pretreatment with the respective specific mitogen-activated protein kinase (MAPK) inhibitors (SB203580, PD98059, and SP600125) and NF κ B inhibitor (Bay-11 to 7082) could block SAA-dependent TF induction. SAA also directly induced activation of MAP kinases and NF κ B.

Conclusions—The stimulating effect of SAA was faster-acting on the expression and activity of TF and the inhibitory effect was slower-acting on TFPI. The effects are mediated through FPRL1, MAP kinases and NF κ B. (*Arterioscler Thromb Vasc Biol*. 2007;27:1645-1650.)

Key Words: SAA ■ tissue factor ■ tissue factor pathway inhibitor ■ FPRL1 ■ MAP kinases ■ NF κ B

Coronary artery disease (CAD) is one of the leading causes of death in affluent societies. Atherosclerosis, which is the pathological basis of CAD, is now considered a chronic inflammatory disease of the vascular wall.¹ Serum amyloid A (SAA) is a major acute phase protein induced during inflammation which could be escalated 100- to 1000-fold.² Accumulating evidence in the past decades have shown that SAA is also associated with CAD. Elevated SAA levels in circulation were found in patients with unstable angina and acute myocardial infarction.³ It has been reported as a significant predictor of CAD risk.⁴ Furthermore, the traditional risk factor of a high-cholesterol diet was reported to increase plasma SAA levels in female LDL-receptor null mice,⁵ as well as in lean insulin-sensitive human subjects.⁶ SAA is normally produced in the liver. However, SAA expression has also been found in human atherosclerotic lesion⁷ and was increased at the site of ruptured plaque in acute myocardial infarction.⁸ These evidences suggest that SAA may play a direct role in CAD. Recent studies have demonstrated that SAA may act as a cytokine to cause proinflammation in cultured endothelial cells, which is the initial step of atherosclerosis.⁹ However, the possible impact of SAA on the other stages of atherogenesis remains obscure.

The initiation of coagulation plays an important role in thrombus formation, which is the major cause of acute

coronary events.¹⁰ The coagulation cascade is initiated as soon as tissue factor (TF) binds activated factor VII (FVIIa), which in turn activates factor X (FX), ultimately leading to thrombin formation. Elevated TF expression has been shown in all stages of atherosclerosis, whereas a very low basal level in normal site is maintained.^{11,12} In vitro studies had demonstrated that TF could be markedly induced by some CAD risk factors, such as cytokines (tumor necrosis factor [TNF] α ,¹³ C-reactive protein [CRP]¹⁴), histamine,¹⁵ and oxidized LDL.¹⁶ Thus, TF is involved in the initiation and propagation of acute coronary syndromes. TF pathway inhibitor (TFPI) is a plasma Kunitz-type serine protease inhibitor, which binds TF/FVIIa complex directly and inhibits the coagulation process.¹⁷ Inhibition of TF by recombinant TFPI was reported to reduce acute thrombus formation in human lipid-rich plaque¹⁸ and in animal injured plaques.^{19,20} Conversely, native TFPI degradation after thrombolysis may enhance procoagulation and leads to early reocclusion after thrombolysis in myocardial infarction.²¹ Furthermore, adenoviral overexpression of TFPI in injured arteries inhibited recurrent thrombosis induced by shear stress without affecting systemic coagulation parameters.^{22,23} Although the function of TFPI has been well determined in the past decade, its regulation is poorly understood to date.

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In this study, we investigated the effects of SAA on endothelial expression and activity of TF and TFPI to shed some light on the obscure role of SAA on hemostasis. In addition, we also examined the signal transduction pathway involved in the SAA function.

Methods

Cell Culture

Primary human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECs) (Clonetics) were cultured in EGM-2MV medium (Clonetics) with full supplements and 5% FBS. HUVECs from passages 4 to 5 and HCAECs from passages 3 to 4 were used in the following experiments.

In SAA treatment studies, cells were cultured to confluence, and SAA (PeproTech) was added to the medium at concentrations of 1 to 20 μ g/mL for 0 to 48 hour. The endotoxin levels in the SAA preparation were confirmed by E-TOXATE test kit (Sigma-Aldrich) to be ≤ 1 EU/ μ g protein. In the receptor blocking study, cells were pretreated with 500 ng/mL of pertussis toxin (PTX) (Sigma-Aldrich) for 1 hour²³ and 10 μ mol/L of peptide WRWWWWWW (1st BASE) for 5 minutes²³ and then treated with SAA 20 μ g/mL for 4 hours and 24 hours. In the mitogen-activated protein kinases (MAPK) and NF κ B pathway study, 4 groups of cells were pretreated with 50 μ mol/L of PD98059 (Sigma-Aldrich), 10 μ mol/L of SB203580, 10 μ mol/L of SP 600125 (ALEXIS Biochemicals), and 10 μ mol/L of BAY-11 to 7082 (Sigma-Aldrich)²⁶ each for 1 hour and then treated with SAA 20 μ g/mL for 4 hour.

Protein Expression Estimation by Western Blot

Thirty-microgram protein samples were loaded and separated by 12% SDS-PAGE gel. Rabbit anti-TF antibody (American Diagnostica Inc), mouse anti-phospho-p38 MAPK (Thr180/Tyr182) antibody, and mouse anti-phospho-JNK (Thr183/Tyr185) were used at a concentration of 1:500 while that of anti-phospho-ERK1/2 (Thr202/Tyr204) antibody (Cell Signaling) was used at 1:1000. Anti-rabbit or anti-mouse secondary antibody coupled to horseradish peroxidase (Cell signaling) was used at a concentration of 1:2000. The blots were developed with the LumiGLO substrate (Cell Signaling) for 1 minute and exposed with Clear Blue X-ray film (Pierce). Bands were quantified by densitometry using a scanner and the Quantity One 4.6.1 software (Bio-Rad). The signals were normalized with β -actin (Sigma-Aldrich).

Measurement of Cellular and Secreted TFPI by ELISA

HUVECs of the same density of 10 000 cells per well were seeded onto the 96-well plates. After an overnight incubation, cells were stimulated with 0 to 20 μ g/mL SAA or 10 ng/mL TNF- α for 4, 24, or 48 hours. The culture supernatants were collected and the TFPI levels were determined by ELISA (Total TFPI IMUBIND, American Diagnostica Inc). All the procedures were carried out according to the manufacturer's manual. Cellular TFPI protein levels were also determined by the same ELISA kit. Sixty microgram of each individual samples were used to measure the TFPI levels after the protocol.

Determination of Gene Expression Levels by Quantitative RT-PCR

Total RNA was isolated using RNAeasy Mini Kits (Qiagen). The LightCycler RNA Master SYBR Green I kits (Roche) were used to quantify the starting mRNA of TF and TFPI. The house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences are listed in the supplemental data, available online at <http://atvb.ahajournals.org>. Following the manufacturer's instructions, 90 ng RNA template, primers, Mn(OAc)₂, and LightCycler RNA Master SYBR Green I were mixed well and transferred into LightCycler capillaries. The program of LightCycler 2.0 system was set up to carry out reverse

transcription, denaturation, amplification, melting curve analysis, and final cooling. The crossing point of each sample was measured and the relative treatment versus control ratio of each target gene was analyzed with LightCycler Software Version 3.5.

TF and TFPI Activity Assays

Actichrome TF activity assay kit (American Diagnostica Inc) was used to determine the TF activity of the cell lysates of HUVECs with or without 20 μ g/mL SAA treatment. Thirty-microgram protein samples were applied to assay the cellular TF activity as described in the manufacturer's manual.

Actichrome TFPI activity assay kit (American Diagnostica Inc) was used to determine the TFPI activity of the culture medium of stimulated and unstimulated HUVECs. All the procedures were carried out according to the manufacturer's manual.

For cell surface TF or TFPI activity, HUVECs of the same density of 10 000 cells/well were seeded onto the 96-well plates and incubated overnight. Cells were treated with SAA 20 μ g/mL for 4 hours or 24 hours. The procedures were as above, except that cell monolayers were washed twice with 1XPBS and reagents were added directly to the cell culture wells.

Nuclear Protein Extraction and NF κ B Transcription Factor Assay

Nuclear protein was isolated using the Nuclear Extraction Kit (Chemicon). The nuclear NF κ B level was measured by TransFactor Profiling Kit-Inflammation 1 (BD Biosciences). Forty micrograms of nuclear extract was applied to the microplate. All the procedures were carried out according to the manufacturer's manual.

Statistical Analysis

Measurements were expressed as mean \pm SD from at least 3 samples. The differences in the effects of the time course and concentration were compared by Mann-Whitney test using SPSS 12.0. The other differences were compared by student *t* test using Microsoft Excel. Significant difference was defined as probability value <0.05 .

Results

SAA Induces TF Expression in Endothelial Cells (ECs)

To examine whether SAA could lead to endothelial procoagulation, TF expression levels were investigated in relation to varying concentration and time exposure to SAA stimulation. Western blot analysis (Figure 1a) shows the induction of endothelial TF protein level by SAA over time. Recombinant human SAA (20 μ g/mL) markedly induced TF expression between 4 to 8 hours. QRT-PCR data (Figure 1b and 1c) confirmed the TF transcription were highly induced between 2 to 8 hours and that the induction was concentration-dependent. The TF transcription in HCAECs after SAA (20 μ g/mL for 4 hours) incubation showed a similar trend (supplemental data).

SAA Inhibits TFPI Expression in ECs

To investigate the effects of SAA on TFPI, its expression pattern in relation to duration of SAA treatment was determined by ELISA and QRT-PCR. The effect of SAA on TFPI secretion over time is shown in Figure 2a. SAA did not significantly inhibit TFPI at 4 hours, but only thereafter. TFPI level in supernatants of SAA (20 μ g/mL) samples was significantly declined by $38.2 \pm 2.2\%$ at 24 hours compared with the nonstimulated controls and $26.0 \pm 3.1\%$ at 48 hours.

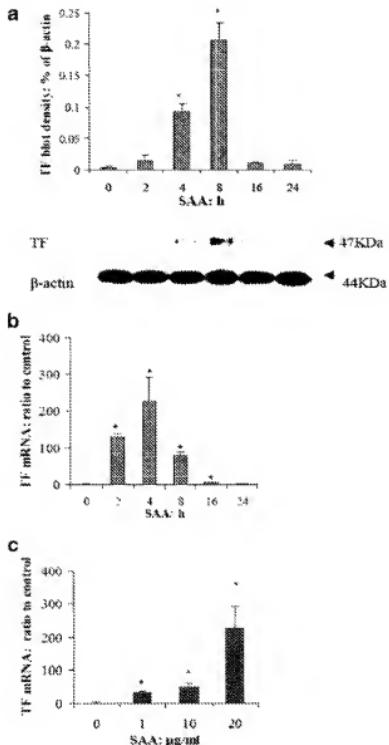


Figure 1. SAA induces TF expression in HUVECs. **a**, SAA (20 μ g/mL) induces TF protein levels. **b** and **c**, Time- and concentration-dependent effects of SAA on TF mRNA. * P <0.05 vs Control. $n=3$ to 4.

The inhibitory effect of SAA on TFPI secretion was concentration-dependent (Figure 2b). Cellular TFPI levels were simultaneously decreased with SAA treatment for 24 hours (Figure 2c). Interestingly, a common cytokine TNF- α (10 ng/mL) showed a similar inhibitory effect on TFPI expression. QRT-PCR confirmed that the gene transcription was also inhibited in longer-term SAA incubation (Figure 2d). Similar gene expression pattern was obtained again in TNF- α samples. As a further confirmation in a different cell line, incubation of HCAECs with SAA (20 μ g/mL) was also shown to inhibit TFPI mRNA level (supplemental data) with a similar time-course trend.

The Effect of SAA on TF and TFPI Activity

Beyond investigation of the effects of SAA on TF and TFPI expression, we proceeded to investigate its effects on TF and

TFPI activity levels as these are crucial in the blood coagulation cascade. Stimulation with SAA (20 μ g/mL) for 4 hours dramatically induced surface TF activity levels up by 26.3 \pm 2.5-fold in HUVECs (Figure 3a). Cellular TF activity in lysate was also induced. Conversely, stimulation with SAA (20 μ g/mL) for 24 hours significantly inhibited cell surface and supernatant TFPI activity (Figure 3c). TNF- α incubation for 24 hours showed the same trends as TFPI activity levels. The time course study of TF and TFPI activity showed a similar pattern as that of protein expression (Figure 3b and 3d).

The Involvement of FPRL1 in SAA Effects

As the effects of SAA have been postulated to be initiated by its binding to specific receptors, we further examined the involvement of FPRL1, which is a member of the G protein-coupled receptor. Pretreatment of HUVECs with PTX, a known inhibitor of the FPRL1, could significantly suppress the TF induction and TFPI inhibition caused by SAA (Figure 4), suggesting the involvement of a G protein-coupled receptor. WRWWWW, which is a novel specific inhibitor for FPRL1, showed a similar blocking effect as PTX (Figure 4). Our results data indicate that FPRL1 is likely to be one of the receptors through which SAA mediates its effects.

The Induction of SAA on TF Is Mediated Through MAPK and NF κ B Pathway

To assess whether the induction of TF by SAA is mediated through MAPK and NF κ B pathway, we examined the effect of specific pathway inhibitors on TF induction. SB203580, PD 98059, SP600125, and Bay-11-7082 are specific inhibitors for p38, ERK1/2, JNK, and NF κ B, respectively. Preincubation with these inhibitors was shown to significantly inhibit SAA-induced TF expression (Figure 5a) and activity (supplemental data). In addition, p38, extracellular signal regulated kinase (ERK), and JNK were transiently activated after stimulation with SAA (20 μ g/mL) (Figure 5b). Moreover, the active subunit NF κ B p65/50 levels in nuclear extraction were significantly elevated after SAA treatment for 0.5 to 1 hour (Figure 5c), indicating that SAA also directly led to NF κ B translocation and activation.

Discussion

Ross proposed in 1999 that atherosclerosis is an inflammatory disease and that inflammatory factors play key roles in atherogenesis.¹ Many classical cytokines have been shown to lead directly to endothelial procoagulation through TF induction. This time-course study has shown that SAA could exert very significant effect on the expression and activity of TF and TFPI in human endothelial cells—inducing TF between 4 to 8 hours and inhibiting TFPI between 24 to 48 hours. The presence of at least 10 μ g/mL of SAA could bring about the hypercoagulable state through either an elevation of endothelial TF or an inhibition of TFPI. These phenomena have not been reported before in earlier studies involving SAA or other common cytokines.

The ability of SAA to induce TF expression is not unexpected. Recent studies have demonstrated that SAA may act as a cytokine and could lead to vascular proinflammation.

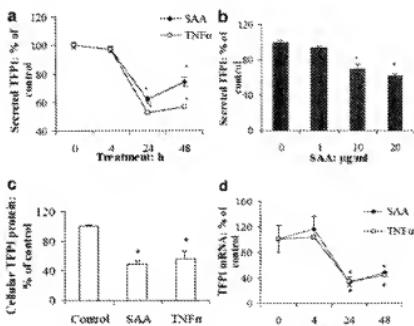


Figure 2. SAA inhibits TFPI expression in HUVECs. **a**, **b**, TFPI secretion in cell supernatants. **c**, **d**, TFPI protein levels in cell lysates, **c**, TFPI mRNA. Cells were incubated with SAA 20 μ g/mL or TNF- α (10 ng/mL) for 24 hours or in a time course. * P <0.05 vs Control or "0". n =3 to 5.

SAA was reported to induce interleukin (IL)-8 in neutrophils²⁴ and cellular adhesion molecules in human endothelial cells.²⁵ As for TF, it is highly sensitive to cytokines such as TNF- α , IL-1 β ,¹⁰ and another acute-phase protein, CRP.¹⁴ Therefore, it is reasonable to speculate that SAA may also stimulate TF expression and activity. A very recent study parallel to our study showed that SAA could induce TF expression in monocytes.²⁷ Our results have shown that that SAA could markedly induce endothelial TF and that the elevation of TF was observed at all 3 different levels of mRNA, protein and activity. The SAA-dependent TF induction appeared after a period of short stimulation (4 to 8 hours), with its effect wearing off after 16 hours. This time-dependent effect is comparable to other stimuli, such

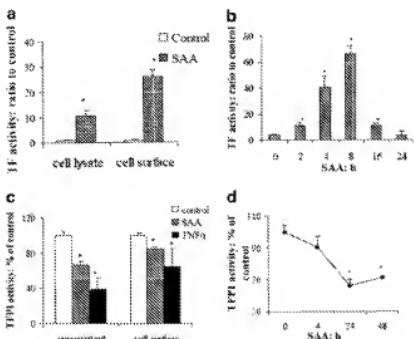


Figure 3. SAA induces TF activity (a, b) and inhibits TFPI activity (c, d). Cells were incubated with SAA 20 μ g/mL for 4 hours (TF activity) or for 24 hours (TFPI activity). TNF- α was used at 10 ng/mL for 24 hours. * P <0.05 vs Control. n =3 to 5.

as TNF- α , IL-1 β , CRP, thrombin, histamine, and oxidized LDL.¹⁰

At the cellular level, TF expression and activity are counterbalanced by TFPI. Inflammatory factors are believed to regulate the expression and activity of TFPI because they have widespread effects on thrombosis, including the balance of coagulation and fibrinolysis. However, these TF inducers did not show clear and consistent impacts on TFPI. Shimokawa et al found that high dose of TNF- α (50 ng/mL) and lipopolysaccharide (LPS) (10 to 100 μ g/mL), but not IL-1 (10 U/mL), could significantly decrease murine TFPI mRNA level in MSS-31 endothelial cells after 2 to 8 hours treatment, with their effects wearing off after 24 hours.²⁸ Szotowski et al found that TFPI was decreased in cell lysate of HUVECs but increased in supernatant after 5 and 18 hours treatment by TNF- α (10 ng/mL) or IL-6 (10 ng/L), suggesting TFPI was secreted out from cells into the supernatant.²⁹ However, they did not measure the TFPI transcription and total protein production. To obtain more comprehensive results, we measured the transcription, protein production, and activity levels of TFPI through a time course of SAA treatment for 0 to 48 hours. Our data demonstrated that SAA could significantly inhibit TFPI expression and secretion with longer treatment (24 to 48 hours), but not with the short treatment (4 hours). Both cellular and secreted TFPI protein levels were simultaneously inhibited by SAA in a concentration-dependent manner, suggesting that such effect was not attributable to protein redistribution from the cells to the supernatant but caused indeed by lower protein synthesis. Our mRNA results confirmed that TFPI transcription was also inhibited after cells were incubated with SAA for 24 to 48 hours. The effects of SAA on both TF and TFPI suggest that the hypercoagulable state could be sustained for at least 48 hours. Such time course effects have not been reported before in earlier studies of SAA and other cytokines. We tested another cytokine, TNF α , and obtained similar sustained effects for 48 hours. The hypercoagulable state induced by SAA may have an important clinical implication as SAA is known to be expressed at the site of atherosclerotic plaque⁷ and elevated after plaque rupture.⁸ SAA-dependent TF induction may contribute to the thrombosis and acute coronary events. On the other hand, inhibited TFPI expression and activity may explain the reocclusion of the coronary arteries after thrombolysis treatment because native TFPI degradation was believed to play a role in reocclusion.

The mechanism of SAA effects have caused increased interests for the past years. Several potential receptors for SAA have been postulated, including scavenger receptor B-I (SR-BI)³⁰ and its human orthologue CLA-1,³¹ receptor for advanced glycation end products (RAGE),²⁷ and FPRL1.²⁴ A very recent study conducted by Cai et al found that the SAA-induced monocyte TF expression was markedly inhibited by anti-RAGE, suggesting the involvement of RAGE.²⁷ Our study demonstrated for the first time that FPRL1 was involved in the SAA-dependent TF and TFPI regulation in human endothelial cell. With the use of an inhibitor of G protein-coupled receptor (PTX), both the TF induction and TFPI inhibition caused by SAA were significantly suppressed. Moreover, a novel specific inhibitor for FPRL1

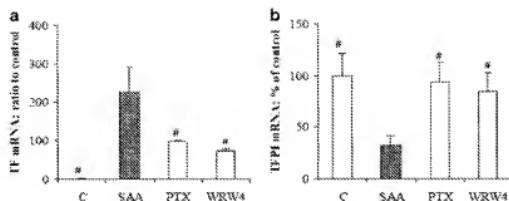


Figure 4. FPRL1 inhibitors block the effects of SAA on TF (a) and TFPI (b). Cells were pretreated with PTX (500 ng/mL) for 1 hour or WRW4 (10 μ mol/L) for 5 minutes, and then incubated with SAA 20 μ g/mL for 4 hours (TF) or for 24 hours (TFPI). * P <0.05 vs SAA group. n=3 to 4.

(WRW4), which was discovered in 2004,²⁵ had similar inhibitory effects. These results suggested that the SAA effects were regulated at least partially by FPRL1. FPRL1 was suggested to be a potential receptor for SAA on phagocytes and mediates its chemotactic activity.³² In 2003, He et al found that SAA-induced IL-8 secretion in neutrophils was triggered by its binding to a special G-coupled receptor, FPRL1.³³ PTX and an antibody against the N-terminal domain of FPRL1 effectively blocked SAA-induced IL-8 secretion. Lipoxin A4, a specific substrate of FPRL1, competitively suppressed SAA-induced IL-8 secretion. More recently, FPRL1 was also found to mediate the SAA-induced matrix metalloproteinase (MMP)-9 expression in human monocytes³³ and PGE₂ production in neutrophils.³⁴ In 2006, SAA was found to stimulate the proliferation of HUVECs and the effects appeared to be mediated by FPRL1, as it was mimicked by a specific ligand for FPRL1, the WKYMVm peptide.³⁵ Our finding extends its range of effects to TF induction and TFPI inhibition in ECs.

The MAP kinases (p38, ERK, and JNK) and NF κ B are involved in endothelial TF induction through stimulation by many agents, such as TNF- α , histamine, and thrombin.¹⁰ This pathway is crucial in inflammatory response because NF κ B is a major transcription factor regulating many target genes, including cellular adhesion molecules, ILs, and TF. NF κ B activation lies downstream of MAPK activation which is triggered by many of the stimuli proposed to be involved in

atherosclerosis, such as modified lipoproteins, cytokines, and infectious agents.³⁶ Activation of p38, ERK, and NF κ B were reported in SAA-stimulated neutrophils,²⁴ human monocytic THP-1 cells,³¹ and impacted on IL-8 secretion. Very recently, Cai et al found that SAA induced TF expression through MAPK and NF κ B activation in monocytes.²⁷ Our study showed that specific MAPK inhibitors and NF κ B inhibitor could block the SAA-induced TF expression. Furthermore, results from Western blot demonstrated that SAA directly activated p38, ERK, and JNK by rapid phosphorylation. Our study has also shown that SAA could induce nuclear translocation and activation of NF κ B. These results suggest that SAA-induced TF expression is mediated through the MAPK and NF κ B activation.

In summary, this study has investigated the direct roles of SAA on procoagulation. SAA was found to rapidly induce TF expression and activity, whereas the inhibitory effect on TFPI was more slow acting. The effects were mediated through FPRL1, MAPK, and NF κ B. These findings suggest that SAA may have implications in CAD and could be a potential novel target for CAD treatment.

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Disclosures

None.

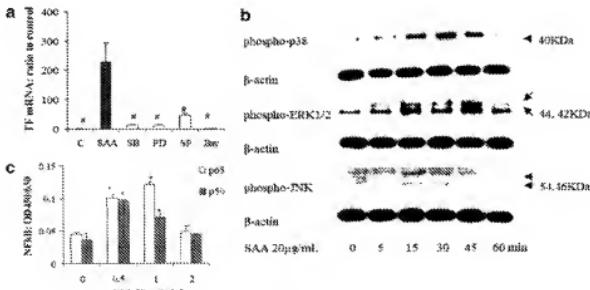


Figure 5. The induction of SAA on TF expression is mediated by MAPK and NF κ B. SB203580 (10 μ mol/L), PD98059 (50 μ mol/L), SP600125 (10 μ mol/L), and Bay-10-7082 (10 μ mol/L) inhibit the SAA-induced TF expression (a). b, SAA induces p38 MAPK, ERK1/2 and JNK phosphorylation. c, SAA induces active subunit NF κ B p65/p50 levels in nuclear extraction. * P <0.05 vs Control. # P <0.05 vs SAA group. n=3 to 4.

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